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Simple diffusion delivery via brain interstitial route for the treatment of cerebral ischemia

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Delivering pharmacologic agents directly into the brain has been proposed as a means of bypassing the blood brain barrier. However, despite 16 years of research on a number of central nervous system disorders, an effective treatment using this strategy has only been observed in the brain tumor glioblastoma multiforme. Within this study we propose a novel system for delivering drugs into the brain named the simple diffusion (SDD) system. To validate this technique, rats were subjected to a single intracranial (at the caudate nucleus), or intraperitoneal injection, of the compound citicoline, followed two hours later by a permanent middle cerebral artery occlusion (pMCAO). Results showed that 12 h after pMCAO, with 0.0025 g kg^{-1} citicoline, an infarct volume 1/6 the size of the intraperitoneal group was achieved with a dose 1/800 of that required for the intraperitoneal group. These results suggest that given the appropriate injection point, through SDD a pharmacologically effective concentration of citicoline can be administered.

permanent middle cerebral artery occlusion, animal model, brain ischemic injury, citicoline therapy, MRI, neuroprotection

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A major obstacle in the development of drugs for use in the central nervous system is the ability to generate compounds capable of penetrating the blood brain barrier (BBB) [1]. One strategy, developed by Oldfield *et al*. [2], to overcome this problem is to bypass the BBB and infuse drugs directly into the CNS parenchyma through a process known as convection-enhanced delivery. With this technique a constant pressure gradient is maintained during interstitial infusion via one or several catheters and there is significantly enhanced distribution of a range of molecule sizes and an increase in the locoregional concentration of the infused compounds. Despite 16 years of basic research and multiple clinical trials using this technique being carried out on various CNS diseases [3], effective treatment has only been obtained in the brain tumor glioblastoma multiforme [4].

In this report, we outline the findings of a novel simple diffusion delivery (SDD) system used for the treatment of cerebral ischemia. This system is based on previous studies examining brain interstitial tracer imaging using MRI [5] wherein, we demonstrated that within two hours, using the caudate nucleus as the original injection point, small molecules like Gd-DTPA are able to diffuse via the brain extracellular space to most of the architecture supplied by the middle cerebral artery. The compound Gd-DTPA is a common MRI contrast agent, which is also an effective tracer for monitoring the dynamic distribution of small molecules in the interstitial space [6]. Based on the above findings, we sought to test the hypothesis that intracerebral

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administration of the compound citicoline prior to ischemic injury would provide a more efficient neuroprotective effect than other routes of administration. In the current study, citicoline was chosen to test our hypothesis because it has a similar polarity and molecular weight to Gd-DTPA [7,8].

1 Materials and methods

1.1 Animals

The study was conducted in accordance with national guidelines for the care and use of animals. All animal protocols were approved by the Peking University Health Science Center Ethics Committee (Approval No. LA2009-008). The animals used in the study were age-matched, mature male Sprague Dawley rats weighing approximately 250–300 g.

1.2 Experimental groups

Citicoline (CDP-choline, cytidine 5′-diphosphocholine) was administered by intracranial microinjection into the caudate nucleus using the following stereotactic coordinates: bregma +1 mm, lateral 3 mm, vertical 4.5 mm (Lab Standard Stereotaxic-Single, Stoelting Co., Illinois, USA). The study of the real-time detection of the infarction process by MRI and determination of infarct volume by TTC staining was composed of seven groups. For each group the animals (n= 8/group) received an intracranial (IC) microinjection of (i) 5 μL saline (control group), (ii) 0.00125 g kg^{-1} citicoline (IC group 1), (iii) 0.0025 g kg^{-1} citicoline (IC group 2), (iv) 0.00375 g kg^{-1} citicoline (IC group 3), (v) 0.005 g kg^{-1} citicoline (IC group 4), (vi) 0.0125 g kg^{-1} citicoline (IC group 5). Animals in the final group received an intraperitoneal (IP) injection of 2 g kg^{-1} citicoline (IP group). Two hours after receiving an injection of citicoline (or saline), the animals were then subjected to a permanent middle cerebral artery occlusion (pMCAO). The choice to administer citicoline two hours prior to pMCAO was based on our previous study on the diffusion pattern of Gd-DTPA in the brain extracellular space [5].

1.3 Permanent middle cerebral artery occlusion (pMCAO) in rats

The rats were anesthetized with a compound anesthetic agent (sodium pentobarbital, ethanol, chloral hydrate, magnesium sulfate, propylene glycol) delivered intraperitoneally. The animals' core temperatures were monitored with a rectal thermometer and maintained at approximately (38±0.5)°C using a heat pad. Permanent focal cerebral ischemia was induced using a technique modified from work presented by Longa *et al*. [9] without the process of vascular recanalization for perfusion. After the surgery, all animals were returned to their cages and allowed free access to water and food.

1.4 MRI for pMCAO rats

Neuroimaging was performed using a 3.0T clinical MR system (Magnetom Trio, Siemens Medical Solutions, Erlangen, Germany). A wrist coil was used for axial T_1W, T_2W and DW images. Parameters for the imaging sequence were as follows: (i) 3D MP-RAGE T_1WI (echo time (TE)= 3.7 ms; repetition time (TR)=1500 ms; flip angle α=9°; inversion time (TI)=900 ms; field of view (FOV)=267 mm; voxel=0.5 mm×0.5 mm×0.5 mm; resolution=512×512); (ii) TSE-T_2WI (TE=92 ms; TR=3620 ms; flip angle α=120°; slice thickness, 2 mm; FOV=80 mm; resolution=256×256); (iii) FSIP-DWI (TE=6.22 ms; TR=15.3 ms; flip angle α=30°; slice thickness, 1 mm; FOV=80 mm; voxel=0.4 mm×0.4 mm×1.0 mm; diffusion moment=0 and 50 mT m^{-1} ms^{-1}; resolution=192×192). MRI scanning was performed 12 h after the pMCAO surgery and prior to sacrifice.

1.5 Neurological deficit evaluation

A thorough neurologic examination was performed on all animals after surgery. The neurologic findings were scored along a five-point scale based on the Zea-Longa scale [7] whereby zero indicated no neurologic deficit, a score of one (characterized by the failure to extend left forepaw fully) indicated a mild focal neurologic deficit, a score of two (characterized by circling to the left) indicated a moderate focal neurologic deficit and a score of three (characterized by falling to the left) indicated a severe focal deficit. Rats with a score of four did not walk spontaneously and had a depressed level of consciousness.

1.6 TTC staining and infarct volume determination

Twelve hours after performing the pMCAO surgery the animals were sacrificed. The brains were removed and series of 2 mm of coronal brain slices were collected (Brain Matrix, EHSY, Shanghai, China) and stained for 30 min at 37°C in 0.2% TTC (2,3,5-triphenyl-tetrazolium chloride, Sigma, Missouri, USA) diluted in 0.1 mol L^{-1} phosphate buffer [10]. The infarct volume was measured by recording stained sections with a digital camera (Canon IXUS800, Canon, Tokyo, Japan) and the image of each section was analyzed by an image analyzer (Image Pro Plus 6.0, Media Cybernetics, Inc., Georgia, USA). The infarct area, which was not stained, was determined by counting pixels within the outlined regions of interest. Infarct volume was expressed as the ratio of pixels counted from the corrected infarct area to the pixels collected from the whole contralateral hemisphere. We then compared the infarct regions with those derived from MRI.

1.7 Statistical analysis

Values are presented as mean±SD. Statistical analysis was performed using SPSS 13.0. One-way analysis of variance followed by individual comparisons of means (LSD test, or Dunnet's method when the data were not normally distributed) was used for the comparison of grouped data. Data showing $P<0.05$ were considered statistically significant.

2 Results

2.1 Intracranial administration of citicoline reduces infarct volume and neurological deficit of brain ischemic injury

The results showed that IC administration of citicoline prior to pMCAO had a neuroprotective effect. These effects were confirmed at both the pathological (via TTC staining) and functional levels (via a Zea-Longa five-point scale). The best effect was observed in IC group 2 relative to all other groups ($P<0.01$, Table 1 and Figures 1 and 2). There was no statistical difference between IC groups 1, 3, 4 and 5, the IP group and the control group ($P>0.05$, Table 1 and Figures 1 and 2). On the other hand, there was a statistical difference between IC group 2 and the IP group ($P<0.01$, Table 1 and Figures 1 and 2). As observed in a previous study, behavioral deficits were correlated to the severity of the damage in the cortex [9]. Therefore, the improved neural function might be attributed to a rescue of the penumbra in external cortex by citicoline administration, which was confirmed by the MRI findings (Figure 2).

2.2 MRI results at 12 h after pMCAO

We also used MRI to monitor the pathological progression of all groups during ischemia. Generally, T_1WI is good for anatomical imagine whereas T_2WI is known to be highly sensitive to water content changes. The typical MRI changes associated with infarction, including decreased signal intensity on T_1WI and increased signal intensity on T_2WI [11], are presented to define the lesions for both localization and injury severity (Figure 2).

The TTC staining and MRI showed different localizations of ischemic lesions in six of the groups. The typical changes of decreased signal intensity on T_1WI and increased signal intensity on T_2WI, were well displayed in the infarct areas. Each group consisted of $n=8$ animals.

3 Discussion

In the present work, we have developed a simple diffusion delivery (SDD) technique, which has the potential to distribute the pharmacological agents more efficiently and less invasively within the brain parenchyma. Using this technique, a significant neuroprotective effect against cerebral ischemia can be achieved with a single injection of only a very small dose of the agent. The efficiency of SDD is based on the original injection point and the neuroprotective agents used.

The design of the SDD system was based on brain interstitial MRI tracer imaging using the gadolinium derivative Gd-DTPA whereby MRI can be used to trace the diffusion and distribution of Gd-DTPA within the brain interstitial space. Our previous study found that using the caudate nucleus as the original injection point a single administration Gd-DTPA was able to diffuse to the MCA territory and where it remained almost unchanged for 2–4 h after the injection [5]. In the current work, we assumed that a neuroprotective agent similar to Gd-DTPA would diffuse in the same way as Gd-DTPA. Among the many candidates, citicoline was chosen because it is similar to Gd-DTPA in respect to both molecular weight (510.31 Da vs. 938.02 Da) and polarity (both are hydrophilic). Additionally, because citicoline is an intermediate in the generation of phosphatidylcholine from choline in the normal brain, it is likely to be safer and more appropriate than other exogenous compounds for this local intracranial drug delivery study [12].

Citicoline is considered to be one of the most promising candidates for the treatment of acute ischemic stroke [13–16]. Its functions include accelerating resynthesis of phospholipids and suppressing the release of free fatty acids, stabilizing cell membranes and reducing free radical generation [17]. However, the extremely low brain uptake of citicoline is one of the major obstacles affecting its efficacy as demonstrated in meta-analysis of clinical trials assessing the concentration-dependent neuroprotective effects of citicoline [18]. More importantly, the limited volume fraction of brain microvessels (3% of the total brain volume) [19], and the post-ischemic hypoperfusion state before successful vascular recanalization therapy also make the infusion of citicoline to the infarct area even more difficult. In the present work, using SDD after a single administration of small dose of citicoline resulted in significant protective effects. Our results indicate that citicoline successfully circumvented the BBB and suggests that the issue of poor delivery

Table 1 Neurological findings (based on a five-point Zea-Longa score) and infarct volume ratio observed 12 h after pMCAO surgery[a)]

Groups	Control group (saline)	IC group 1 (0.00125 g kg^{-1} citicoline)	IC group 2	IC group 3	IC group 4	IC group 5	IP group
Neurological score	2.9±0.8	2.9±0.8	1.6±0.9	1.9±0.4	2.0±1.2	1.7±0.6	2.0±0.8
Infarct volume ratio (%)	27.7±10.5	27.5±11.9	4.1±2.0	24.0±12.9	23.5±11.7	30.3±18.8	24.0±10.4

a) Animals from IC group 6 did not survive 12 h and were not included. For each group $n=8$ rats.

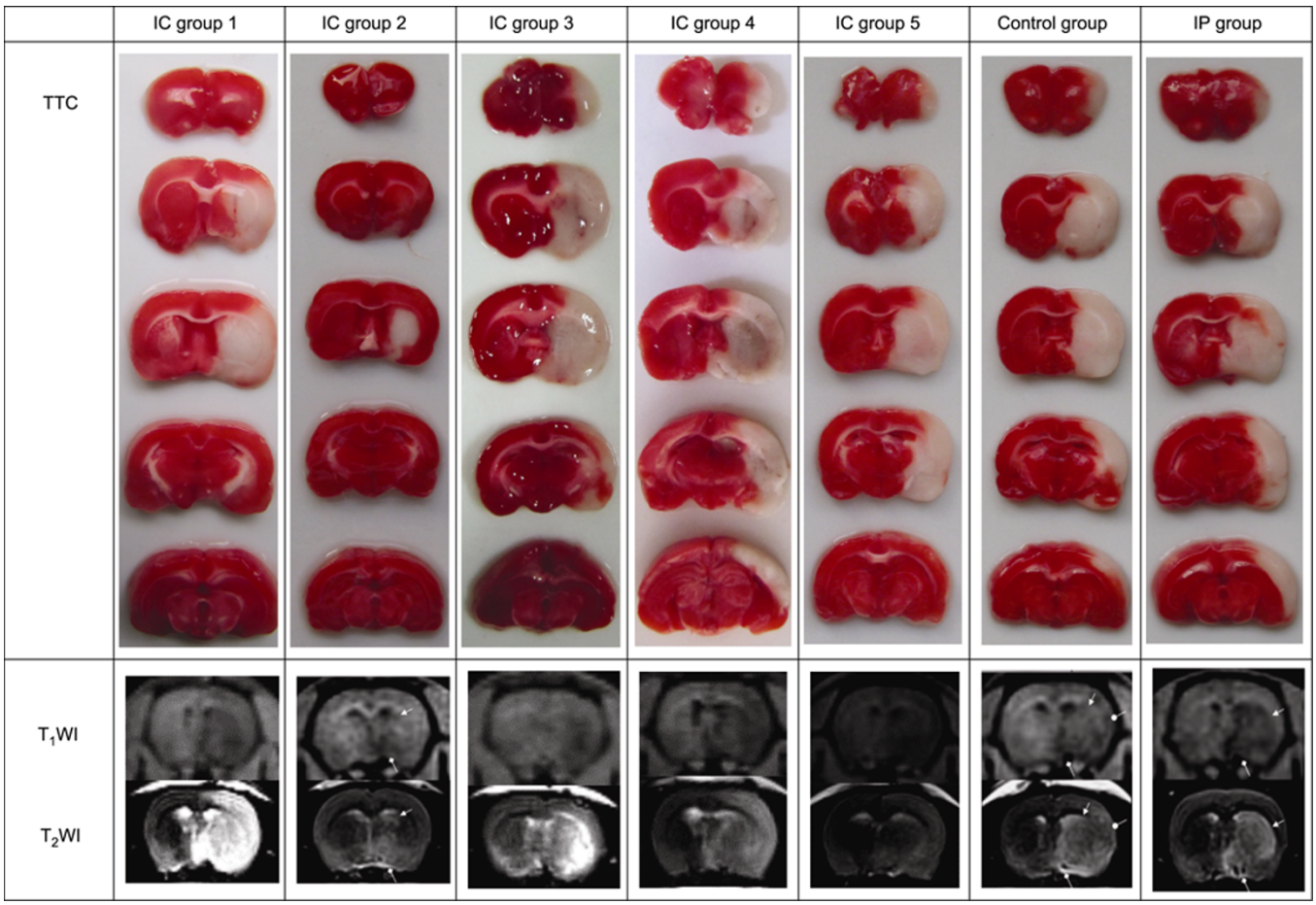

Figure 1 TTC staining and MRI results. Each group, except group 7, received an IC microinjection containing 5 μL saline (group 1, control group), 0.00125 g kg^{-1} citicoline (group 2, IC group 1), 0.0025 g kg^{-1} citicoline (group 3, IC group 2), 0.00375 g kg^{-1} citicoline (group 4, IC group 3), 0.005 g kg^{-1} citicoline (group 5, IC group 4), and 0.0125 g kg^{-1} citicoline (group 6, IC group 5). Animals from group 7 (IP group) received an IP injection of 2 g kg^{-1} citicoline. For each group, two hours following injection, the rats received a permanent middle cerebral artery occlusion (pMCAO).

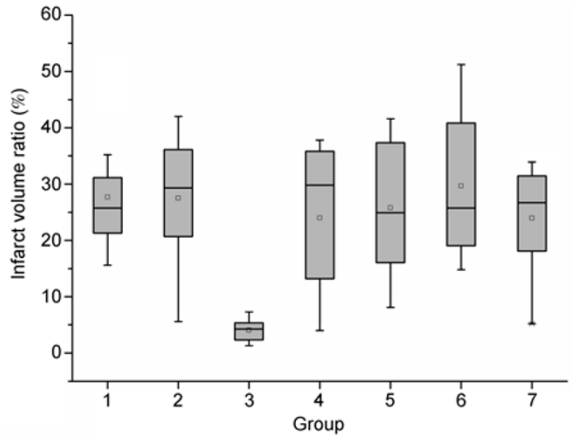

Figure 2 The infarct volume ratio in the seven groups. Each group, except group 7, received an IC microinjection containing 5 μL saline (group 1, control group), 0.00125 g kg^{-1} citicoline (group 2, IC group 1), 0.0025 g kg^{-1} citicoline (group 3, IC group 2), 0.00375 g kg^{-1} citicoline (group 4, IC group 3), 0.005 g kg^{-1} citicoline (group 5, IC group 4), and 0.0125 g kg^{-1} citicoline (group 6, IC group 5). Animals from group 7 (IP group) received an IP injection of 2 g kg^{-1} citicoline. For each group, two hours following injection, the rats received a permanent middle cerebral artery occlusion (pMCAO). Each group consisted of n=8 animals.

due to hypoperfusion may have also been overcome.

In summary, we have developed a novel delivery technique for the treatment of CNS diseases. Compared with traditional techniques such as convection-enhanced delivery, SDD is more efficient and less invasive. Although the MRI tracer imaging enables a precise predictive control over the SDD process in our study, a reliable quantitative measurement on the diffusion and clearance properties within brain interstitial space is the key to understanding the mechanism of SDD and enhancing its application for the treatment of CNS diseases.

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